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# PATENT APPLICATION

# SYSTEMIC DELIVERY OF SERUM STABLE PLASMID LIPID PARTICLES FOR CANCER THERAPY

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PATENT

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# SYSTEMIC DELIVERY OF SERUM STABLE PLASMID LIPID PARTICLES FOR CANCER THERAPY

## CROSS-REFERENCES TO RELATED APPLICATIONS

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~~This application claims the benefit of U.S. Provisional Patent Application Serial Nos. 60/101,429, filed September 22, 1998 and 60/086,917, filed May 27, 1998, which are incorporated herein by reference in their entirety for all purposes. This application is also related to U.S. Patent Application Serial No. _____, filed February 2, 1999 (Attorney Docket No. 016303-006220), and claims the benefit of U.S. Provisional Patent Application Serial Nos. 60/112,384, filed December 14, 1998, and 60/073,598, filed February 3, 1998, which are incorporated herein by reference in their entirety for all purposes.~~

## FIELD OF THE INVENTION

This invention relates to methods and compositions for treating a neoplasia and, in particular, a tumor in a mammal.

## BACKGROUND OF THE INVENTION

Systemic delivery of therapeutic nucleic acids for gene therapy applications is a highly desirable goal. Systemic delivery using blood or lymph circulatory systems will permit therapeutic nucleic acids to seek out multiple distal disease sites, and to approach a disease site from multiple points. Successful systems will be useful against cancers (*i.e.*, solid, nonsolid or metastatic tumors), infectious diseases, inflammation and other disease targets that are distal to the site of administration.

Currently, the most popular working systems for *in vivo* human gene therapy are not systemic delivery systems. Current systems employ direct, *i.e.*, local, injection or inhalation of modified adenoviruses (*see*, Englehardt, "Methods for Adenovirus-Mediated Gene Transfer to Airway Epithelium," Chapter 11 in Methods in Molecular Medicine, Gene Therapy Protocols. Ed. P. Robbins, 1997. Humana Press Inc., Totowa, NJ), retroviruses (Olsen, *et al.*, "Methods for the Use of Retroviral Vectors

for Transfer of the CFTR Gene to Airway Epithelium," Chapter 10, Methods in Molecular Medicine, *supra*), cationic lipid-plasmid aggregates (Nabel, *et al.*, "Methods for Liposome-Mediated Gene Transfer to Tumor Cells *in vivo*," Chapter 21, Methods in Molecular Medicine, *supra*; Son, *et al.*, "Cationic Liposome–Mediated Gene Transfer to Tumor Cells in Vitro and *In vivo*," Chapter 23, Methods in Molecular Medicine, *supra*), or simply delivery of naked DNA (*see*, U.S. Patent No. 5,589,466 to Felgner, *et al.*).

Unfortunately, there are well-known drawbacks to these popular methods that prevent their use as systemic delivery systems. For instance, cationic lipid-plasmid aggregates are rapidly cleared by the liver, lung or spleen after intravenous delivery and, therefore, do not satisfy the criteria for systemic delivery since they demonstrate little useful transfection elsewhere. This may be a result of their large diameter, *i.e.*, >200 nm, and powerful cationic surface charge. Moreover, adenoviral and retroviral systems are immunogenic, are rapidly cleared from circulation and do not permit repeat dosing. In addition, naked DNA is rapidly degraded in the blood and, thus, is not suitable for systemic delivery.

Systemic delivery for *in vivo* gene therapy, *i.e.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, a less well explored avenue, has been achieved using lipid-plasmid particles such as those disclosed in published PCT Patent Application WO 96/40964, U.S. Patent No. 5,705,385, and U.S. Patent Applications Serial Nos. 08/485,458, 08/484,282, 08/660,025, 08/856,374, 60/073,598 and 60/063,473, all of which are assigned to the assignee of the instant invention and incorporated herein by reference. This latter format provides a fully encapsulated lipid-plasmid particle that protects the therapeutic nucleic acid from nuclease degradation in serum, is nonimmunogenic, is small in size and is suitable for repeat dosing.

Once the systemic delivery system has been established, the next question is to determine which disease condition to treat and which therapeutic nucleic acid to deliver. To date, no publication has reported therapeutic data employing, in a systemic delivery system, the variation of the gene therapy technique known as gene-delivered enzyme prodrug therapy ("GDEPT") or, alternatively, the "suicide gene/prodrug" system, which was first developed by Moolten, F.L., Cancer Res., 46:5276-5281 (1986). For a review of the GDEPT system, *see* Moolten, F.L., The Internet Book of Gene Therapy, Cancer Therapeutics, Chapter 11 (Sobol, R.E., Scanlon, NJ (Eds) Appelton & Lange

(1995)). In this method, a heterologous gene is delivered to a cell, the heterologous gene encoding an enzyme that promotes the metabolism of a first compound to which the cell is less sensitive (*i.e.*, the "prodrug") into a second compound to which is cell is more sensitive. The prodrug is delivered to the cell either with the gene or after delivery of the gene. The enzyme will process the prodrug into the second compound and respond accordingly. A suitable system proposed by Moolten is the herpes simplex virus - thymidine kinase (HSV-TK) gene and the prodrug ganciclovir. This method has recently been employed using cationic lipid-nucleic aggregates for local delivery (*i.e.*, direct intra-tumoral injection), or regional delivery (*i.e.*, intra-peritoneal) of the TK gene to mouse tumors by Zerrouqui, *et al.*, *Can. Gen. Therapy*, 3(6):385-392 (1996); Sugaya, *et al.*, *Hum. Gen. Ther.*, 7:223-230 (1996) and Aoki, *et al.*, *Hum. Gen. Ther.*, 8:1105-1113 (1997). Human clinical trials using a GDEPT system employing viral vectors have been proposed (*see*, *Hum. Gene Ther.*, 8:597-613 (1997), and *Hum. Gene Ther.*, 7:255-267 (1996)) and are underway.

Patent applications relating to the GDEPT method have been published under the following numbers: WO 97/19180; WO 97/07118; WO 96/22277; WO 97/19183; WO 96/16179; WO 96/03515; WO 96/03515; WO 96/03151; EP 690129; EP 657541; EP 657539; WO 95/05835 and EP 415731.

From the foregoing, it is readily apparent that systemic delivery of therapeutic nucleic acids to distal disease sites would clearly provide significant advantages over existing gene therapy modalities. It is an object of this invention to provide methods and compositions to achieve this goal.

## SUMMARY OF THE INVENTION

The present invention provides, *inter alia*, methods and compositions for treating a neoplasia, *e.g.*, a tumor, in a mammal. In one embodiment, the present invention provides a method of treating a neoplasia in a mammal, the method comprising administering to the mammal a serum-stable nucleic acid-lipid particle comprising a nucleic acid that is fully encapsulated within a lipid, wherein the administration is by injection at an injection site that is distal to the neoplasia in the mammal.

In another embodiment, the present invention provides a method for sensitizing a neoplastic cell, the method comprising: (a) transfecting the neoplastic cell with a serum-stable nucleic acid-lipid particle comprising a nucleic acid that is fully

encapsulated within a lipid and that encodes a gene-product that promotes the processing, *i.e.*, conversion, of a first compound (*e.g.*, a prodrug) into a second compound, wherein the administration is by injection at an injection site that is distal to the neoplasia in the mammal; and (b) delivering to the neoplastic cell the first compound, wherein the cell is more sensitive to the second compound than to the first compound.

In the above methods, the nucleic acid and the first compound can be delivered in lipid formulations that can be the same or different. The lipid formulations, whether used to deliver the nucleic acid or first compound (*e.g.*, prodrug), can be prepared from a variety of lipids, lipid conjugates and additional compatible components known in the art. The lipid formulations can be prepared, for example, from sphingomyelin and cholesterol. Moreover, the lipid formulations can contain additional components that improve the properties or characteristics of the formulations, such as leakiness, longevity in circulation, reduced toxicity, encapsulation efficiency, *etc.* Such components include, for example, cationic lipids, ATTA-lipid conjugates, PEG-lipid conjugates, targeting agents, *etc.* Once prepared, the lipid-nucleic acid formulations and/or lipid-prodrug formulations can be administered or delivered to the mammal using a variety of techniques known to those of skill in the art. In a preferred embodiment, the lipid-nucleic acid formulation is delivered systemically (by, for example, intravenous injection), whereas the lipid-prodrug formulations can be delivered systemically or regionally or locally.

Any nucleic acid useful in treating neoplasia in a mammal can be administered using the compositions and methods of the present invention. For instance, when the GDEPT system is employed, the nucleic acid can be any nucleic acid that encodes a gene product that promotes the processing, *i.e.*, conversion, of a first compound (*e.g.*, a prodrug) into a second compound to which the mammal or cell of interest is more sensitive or receptive. Examples of suitable gene-products include, but are not limited to, herpes simplex virus thymidine kinase, cytosine deaminase, xanthine-guaninephosphoribosyl transferase, purine nucleoside phosphorylase, cytochrome P450 2B1 and their analogs. Other gene-products suitable for use in the methods of the present invention will be readily apparent to those of skill in the art.

In a preferred embodiment, the first compound is a prodrug, *i.e.*, a compound to which the cell of interest in not initially sensitive to, but which the gene-product converts into a compound to which the cell of interest is more sensitive.

Examples of suitable prodrugs include, but are not limited to, ganciclovir, acyclovir, bromovinyldeoxyuridine, 5-fluorocytosine, 6-thioxanthine, MeP-dr and cyclophosphamide. Other prodrugs suitable for use in the methods of the present invention will be readily apparent to those of skill in the art.

In another embodiment, the present invention provides a method of sensitizing a cell to a compound, the method comprising: a) delivering to a cell an enzyme which promotes the processing of a first compound into a second compound; and b) delivering to the cell the first compound in a lipid formulation; wherein the cell is more sensitive to the second compound than the first compound. In a presently preferred embodiment, both the enzyme and the first compound are delivered in lipid formulations.

In yet another embodiment, the present invention provides a composition for treating a neoplasia in a mammal, the composition comprising a nucleic acid in a lipid formulation and a pharmaceutically acceptable carrier. In a presently preferred embodiment, the composition further comprises a prodrug in a lipid formulation.

In still another embodiment, the present invention provides a kit for the treatment of a neoplasia in a mammal, the kit comprising: a) a nucleic acid in a lipid formulation; and b) a prodrug in a lipid formulation.

In addition, the present invention provides methods for preparing lipid formulated nucleic acids (*e.g.*, vectors and enzymes) and prodrugs that can be used in carrying out the methods of the present invention.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** illustrates the relationship of citrate concentration in the dialysis buffer and the DODAC mol% in the lipid for the preparation of lipid-plasmid particles. The solid dots represent good quality formulations having high association efficiencies (>40%), small size (<100 nm) and low values of size polydispersity (chi-square less than 10, preferably less than 3) on a NICOMP particle sizer. The stars represent formulations containing aggregates having large polydispersity values; and the open circles represent formulations having low association efficiencies (<40%). Proper tuning of the citrate buffer concentration to the cationic lipid charge appears to improve the formulation.

Alternative anionic buffers can also be used if the counterions can prevent the cationic lipid from aggregating during the detergent removal step.

**Figure 2** illustrates the biodistribution of 303i in various organs (*i.e.*, blood, spleen and liver) in C57-Lewis Lung mice.

**Figure 3** illustrates the accumulation of 303i at the tumor site in C57-Lewis Lung mice.

**Figure 4** illustrates a time course of gene product activity at distal (metastatic) tumor sites.

**Figure 5** illustrates gene expression in LS180 tumors (dose response of 303i after 48 hours).

**Figures 6(A) and 6(B)** illustrate the pattern of HSV-TK gene expression within peritoneal tumors.

**Figure 7** illustrates the pINEX-TK10 construct which consists of a pBR322 derived plasmid containing a CMV promoter linked to a "hyper" HSV-TK gene, bovine growth hormone polyadenylation sequence and kanamycin resistance gene.

**Figure 8(A)** illustrates *in vivo* efficacy studies using a tumor model.

**Figure 8(B)** illustrates a 16-day treatment regimen on test mice after tumor inoculation.

**Figure 9(A)** illustrates the *in vivo* gene expression protocol.

**Figure 9(B)** illustrates an assessment of the tumor growth, with the empty formulation showing the largest tumor volume.

**Figure 9(C)** illustrates the efficacy of the suicide gene SPLP of this invention.

**Figure 10** illustrates the long-term survival following treatment with the suicide gene SPLP (*i.e.*, Formulation 1.1 or, alternatively, INEX 303 or 303i) of this invention.

**Figure 11** illustrates the efficacy of systemic delivery of TK303 in the BALB/c CT26 tumor model, *i.e.*, a colorectal tumor model.

**Figure 12** illustrates the effect of the TK FS construct and TK on tumor volume.

## DEFINITIONS

"Sensitizing," as used herein, refers to the ability to increase the sensitivity of a designated system, such as a cell. This term includes changing a cell to make it responsive or more responsive to a compound to which it previously was not responsive, sensitive or was less sensitive. Sensitizing and "more sensitive" also includes changes to a cell such that exposure to a previously nonkilling substance results in cell death.

"Nucleic acid vector" or "vector," as used herein, refers to a composition comprising a nucleic acid sequence encoding a gene product. This is usually a plasmid or viral genome, but can also include other compositions, such as linear nucleic acids, protein/nucleic acid conjugates, *etc.* Depending on the use, vector can also refer to a nucleic acid delivered in a virus encapsulated or protein coated format, wherein the entire composition is known as a vector.

"Neoplasia," as used herein, refers to any aberrant growth of cells, tumors, malignant effusions, warts, polyps, nonsolid tumors, cysts and other growths. A site of neoplasia can contain a variety of cell types, including neoplastic cells, which harbor deleterious genetic mutations, normal cells which are induced by neoplasia, such as vascular endothelia, or immune system cells, such as macrophages and leukocytes, *etc.*

"Therapeutically effective amount," as used herein, refers to an amount that is sufficient or necessary to give rise to a desired therapeutic effect. The therapeutic effect can be obtained directly or indirectly. For instance, the therapeutic agent can lead to activation of other therapeutic agents or can act in combination with additional therapeutic agents. For neoplasia, a therapeutic effect can be, for example, a reduction in growth, inhibition or reduction in size of the neoplasia or inhibition or reduction of metastasis and other malignant attributes, or other beneficial effects, such as subjective or objective observations of physicians and patients.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript. The RNA transcript can be therapeutic of its own accord as in the case of antisense or ribozyme transcription plasmids, or the RNA transcript can be translated into a polypeptide that is also a gene product.

"Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

"Serum-stable" in relation to lipid/therapeutic nucleic acid particles means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA. Suitable assays include, for example, a standard serum assay or a DNAse assay such as those described in the Example section.

"Systemic delivery," as used herein, refers to delivery that leads to a broad biodistribution of a compound within an organism. Some techniques of administration can lead to the systemic delivery of certain compounds, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of a compound is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the compound is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid/therapeutic nucleic acid particles is preferably obtained by intravenous delivery.

## DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present invention provides methods and compositions suitable for treating a neoplasia with a therapeutic nucleic acid, preferably a plasmid encoding an expressible gene, wherein the therapeutic nucleic acid is encapsulated in a lipid particle and administered at a site distal to the site of neoplasia, preferably intravenously, and delivered to the site of neoplasia by systemic delivery.

The invention takes advantage of lipid-nucleic acid particles, wherein the nucleic acid is fully encapsulated and protected from nuclease degradation, and wherein the particles have a small diameter (50-200 nm) and have other attributes suitable for systemic delivery.

In general, patient therapy using the invention is achieved as follows. First, the particles are prepared using lipids and a therapeutic nucleic acid. Thereafter, the particles are administered to a mammal having a neoplasia, preferably by intravenous injection at a site that is distal to the site of neoplasia. The particles are delivered to the site of neoplasia by the blood, lymph or other internal body fluid. A therapeutically effective amount of particles accumulates at the site of neoplasia, and are taken up by cells at the site. Therapeutic effects are obtained at the site of neoplasia generally by transcription of the nucleic acid that leads either to a therapeutic transcription product

(*i.e.*, a ribozyme or antisense oligonucleotide or other RNA), or to an mRNA that is translated into a therapeutic polypeptide.

The benefits of systemic gene therapy against tumors using lipid/therapeutic nucleic acid particles include, but are not limited to, the following benefits.

First, nucleic acids are typically nontoxic in comparison to other chemotherapeutic agents employed. Second, the lipid carrier systems of the present invention demonstrate preferential accumulation at many tumor sites. This phenomena depends, in part, on the "leakiness" of the neo-vasculature of the tumor. This preferential "passive" targeting can be enhanced by targeting agents attached to the outer lipid monolayer, such as FGF which enhances localization of lipid particles at the tumor site (*see*, *e.g.*, Forum, *et al.*, "Liposome Targeting in Animal Models," L. Huang (Ed.), *Journal of Liposome Research*, 7(4):315-534 (1997)). Third, therapeutic nucleic acids potentially provide more efficient therapeutics than convention drugs because they are capable of replication at the site of disease. The many copies of transcripts or gene products generated reduce the total number of active agents that must be delivered to the cells. Further, therapeutic nucleic acids can be used to deliver a substrate to a cell that can be used to generate polypeptides, which are too expensive to manufacture or too difficult to deliver. Fourth, therapeutic nucleic acids, by leading to synthesis of polypeptides within the disease cell, can be used to deliver polypeptides to sites inside the disease cell to which the polypeptide could not be delivered from exogenous routes. Finally, because of their low toxicity, therapeutic nucleic acids can be suitable candidates for combination therapy with other chemotherapeutic agents. A suitable combination therapy approach is described in U.S. Provisional Patent Application Serial No. 60/082,665 (TTC Attorney Docket No. 16303-007100), which is assigned to the assignee of the instant invention and incorporated herein by reference.

To date, several approaches for introducing nucleic acids into cells *in vivo* have been used. These include liposome based gene delivery, intratracheal instillation, aerosolized gene delivery or direct injection. For example, Debs and Zhu WO 93/12240, Debs WO 92/1108 and Debs U.S. Patent No. 5,641,662 all describe aerosolized gene delivery of lipid DNA complexes to mammals. Similarly, Stribling, *et al.*, *PNAS*, 89:11277-11281 (1992), describe lipid delivery to mice. McLachlan, *et al.*, *Gene Therapy*, 2:614-622 (1995), describe DOTAP-mediated lipid delivery of hCFTR to mice.

Canonico, *et al.*, *Am. J. Respir. Cell Mol. Biol.*, 10:24-29 (1994), and Canonico, *et al.*, *The American Physiological Society*, 415-419 (1994), describe lipofectin-mediated gene delivery of há1AT to rabbits by aerosolized gene delivery. Alton, *et al.*, *Nature Genetics*, 5:135-142 (1993), describe DC-chol:DOPE/ DOTAP-mediated delivery of hCFTR and â-gal by aerosol or tracheal instillation to mice. Capelen, *et al.*, *Nature Medicine*, 1(1):39 (1995), describe delivery of CFTR to the nasal epithelia of Humans using a DC-Chol/DOPE mediated procedure, as does McLachlan, *et al.*, *Gene Ther.*, 3(12):1113-23 (1996). A variety of reports of administration of lipid-DNA complexes by parenteral administration have also been made, including Brigham WO 91/06309, U.S. Patent No. 5,676,954, and Debs and Zhu WO 93/24640. Accordingly, a variety of procedures for transducing cells *in vivo* using lipid-mediated techniques are known. However, such procedures have not been shown to be useful for systemic delivery. Details of preferred formulations of the present invention are given below.

### *A. Therapeutic Nucleic Acids*

The compositions and methods of the present invention are useful for delivering a wide variety of therapeutic nucleic acids. These nucleic acids can encode therapeutic polypeptides (*i.e.*, any therapeutic gene product), or therapeutic polynucleotides (*i.e.*, antisense or ribozyme transcription plasmids). The therapeutic nucleic acids of the invention can be expressible genes, such as those just described, or they can be nucleic acids, which by themselves, induce some form of response, perhaps by immune system stimulation.

For use with the instant invention, the most preferred therapeutic nucleic acids are those which are useful in gene-delivered enzyme prodrug therapy ("GDEPT"). Any suicide gene/prodrug combination can be used in accordance with the present invention. Several suicide gene/prodrug combinations suitable for use in the present invention are cited in Sikora, K. in OECD Documents, Gene Delivery Systems at pp.59-71 (1996), incorporated herein by reference, include, but are not limited to, the following:

| **Suicide Gene Product** | **Less Active ProDrug** | **Activated Drug** |
|---|---|---|
| Herpes simplex virus type 1 thymidine kinase (HSV-TK) | ganciclovir(GCV), acyclovir, bromovinyl-deoxyuridine, or other substrates | phosphorylated dGTP analogs |
| Cytosine Deaminase (CD) | 5-fluorocytosine | 5-fluorouracil |
| Xanthine-guanine-phosphoribosyl transferase (XGPRT) | 6-thioxanthine (6TX) | 6-thioguano-sinemonophosphate |
| Purine nucleoside phosphorylase | MeP-dr | 6-methylpurine |
| Cytochrome P450 2B1 | cyclophosphamide | [cytotoxic metabolites] |
| Linamarase | amygdalin | cyanide |
| Nitroreductase | CB 1954 | nitrobenzamidine |
| Beta-lactamase | PD | PD mustard |
| Beta-glucuronidase | adria-glu | adriamycin |
| Carboxypeptidase | MTX-alanine | MTX |
| Glucose oxidase | glucose | peroxide |
| Penicillin amidase | adria-PA | adriamycin |
| Superoxide dismutase | XRT | DNA damaging agent |
| Ribonuclease | RNA | cleavage products |

Any prodrug can be used if it is metabolized by the heterologous gene product into a compound to which the cell is more sensitive. Preferably, cells are at least 10-fold more sensitive to the metabolite than the prodrug.

Modifications of the GDEPT system that may be useful with the invention include, for example, the use of a modified TK enzyme construct, wherein the TK gene has been mutated to cause more rapid conversion of prodrug to drug (*see*, for example, Black, *et al.*, *PNAS (USA)*, 93:3525-3529 (1996)). Alternatively, the TK gene can be delivered in a bicistronic construct with another gene that enhances its effect. For example, to enhance the “bystander effect” also known as the "neighbor effect" (wherein cells in the vicinity of the transfected cell are also killed), the TK gene can be delivered

with a gene for a gap junction protein, such as connexin 43. The connexin protein allows diffusion of toxic products of the TK enzyme from one cell into another. The TK/Connexin 43 construct has been assembled as follows: CMV promoter; TK gene; internal ribosome entry sequence; and Connexin 43 (prepared by Dr. Clay Flowers, Inex Pharmaceuticals Corp.).

In the second step, the prodrug is delivered to the cells. The prodrug can be the free drug or, alternatively, it can be in a lipid formulation. The use of lipid formulations has many surprising and previously undiscovered advantages over the delivery of free drug in the GDEPT system including, but not limited to, improved targeting to the disease site transfected by the vector, prolonged circulation half-life, increased drug loading, reduced toxicity towards nontarget tissues, improved treatment modalities, such as a single bolus injection as opposed to IV drip, and the like. These advantages overcome the limitations of the prior art GDEPT systems. Further, the liposomal formulation of the prodrug will preferably provide similar biodistribution to a lipid vector formulation, thereby concentrating both the vector and the prodrug at the disease site.

Usually, the vector will be delivered to the target cell in advance of the prodrug in order to allow synthesis of the suicide gene product prior to the arrival of the prodrug. Temporal separation can be obtained either by separate administration of vector and prodrug or, alternatively, by providing the formulations simultaneously, wherein the vector formulation rapidly accumulates at the target site and delivers the vector, and the prodrug formulation accumulates or delivers its payload more slowly. As such, using the compositions and methods of the invention, the vector is delivered to the cell to direct synthesis of the suicide gene product, the cell is thereby sensitized, the prodrug is delivered to the cell, and patient therapy, *e.g.*, reduction of tumor size, inflammation or infectious load and the like, is achieved.

In addition to the GDEPT systems, there exists a very wide variety of therapeutic nucleic acids that can be employed in the instant invention. The nucleic acids can be human, nonhuman (*i.e.*, from any other plant, animal or microorganism) or entirely synthetic (*i.e.*, non-naturally occurring). The nucleic acids can be heterologous or homologous with patient DNA. Since treatment of neoplasia does not necessarily require long term or stable expression of the delivered nucleic acid, genes effective in transient

expression systems, such as toxins or immune stimulatory proteins, are also useful in the methods of the present invention.

When the gene is endogenous, a modified sequence, copy number or transcriptional activity of the gene can be delivered. The gene product can be directly toxic, indirectly toxic or it can induce apoptosis or cell differentiation. In the most preferred system, the gene product of the therapeutic gene will demonstrate low toxicity to nontarget tissues, and high toxicity to the disease site. For example, when delivered in the preferred lipid-nucleic acid particles of the invention, the gene product preferably has greater toxicity to tumor cells than liver or spleen cells, where a large portion of particles are normally cleared. Disease site specificity can also be enhanced by employing tissue/disease specific promoters for gene transcription or translation. Tissue specific promoters, and methods of associating them with therapeutic nucleic acids are known to those skilled in the art.

Heterologous genes can be advantageous because their gene products can also serve to induce an immune response. For example, genes used in a suicide gene/pro-drug system can have this effect. Preferred homologous (endogenous) genes suitable for use in the methods of this invention include, but are not limited to, pro-apoptotic genes; poreifirin; tumor suppressor genes (p53 and the like); cytokines (IL-2, IL-12, IL-15, GM-CSF, *etc.*); Heat Shock Proteins; Immunodominant Ag (or tumor-specific protein genes); genes activated in embryos only; TIMP-2 (tissue inhibitor of metallo proteinase-2) and other metastasis inhibiting proteins; replacement genes, such as CFTR, DMD; LDL-R and the like; and anti-angiogenic genes, such as endostatin or angiostatin (*see*, WO 97/15666; WO 95/29242; Boehm, *et al.*, *Nature*, 309:404-407 (1997); and Kerbel, *et al.*, *Nature*, 309:335 (1997)).

IL-12 is a preferred homologous gene that can be employed as a therapeutic nucleic acid in the instant invention (*see*, Tahara, H. and Lotze, M.T., *Gene Ther.*, 2:96-106 (1995)). A suitable IL-12 plasmid construct for delivery is pNGVL3-mIL12 provided by the National Gene Therapy Vector Laboratory at the University of Michigan (Ann Arbor, Michigan).

Preferred heterologous genes include, but are not limited to, genes used in GDEPT combinations (treatment in conjunction with pro-drugs); ribozymes or transcription plasmids encoding ribozymes or antisense transcripts; toxin genes, such as saporin, ricin, diphtheria toxin and cholera toxin (or any other plant, bacterial or fungal

gene); viral protein genes, such as E1A; mutated E6; SV40 Tag, *etc.* Other heterologous genes suitable for use in the methods of the present invention will be readily apparent to those of skill in the art.

Methods of constructing plasmids or other vectors that carry the therapeutic nucleic acids disclosed herein are well known to those skilled in the art (*see, e.g.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.* (eds.) 1995, the teachings of which are incorporated herein by reference). Therapeutic activity can be enhanced by the addition of transcription or translation promoters and other nucleic acid elements, again, all of which are known to those skilled in the art.

Lipid-nucleic acid and the lipid-prodrug formulations can be achieved by any prior art method. The preferred methods result in a high efficiency encapsulation, thereby reducing wastage and expense of the formulation. Lipid-nucleic acid and the lipid-prodrug formulations can be synthesized using standard freeze-thaw and extrusion techniques disclosed by Hope, *et al.*, *Biochim. Biophys. Acta*, 812:55-65 (1985). In addition, other drug loading and encapsulation techniques that can be used are disclosed in U.S. Patent Application Serial Nos. 08/399,692, 08/607,614, 08/588,542, 08/741,622, the teachings of which are incorporated herein by reference. Sizing of the lipid formulations can be achieved using extruders, pressure cells, and other tools known to those of skill in the art.

The possible lipid components of the lipid-nucleic acid and lipid-prodrug formulations of the invention include those components typically used in the art. For instance, sphingosomes, which are disclosed in U.S. Patent No. 5,543,152, and U.S. Patent Applications Serial Nos. 08/536,584, 08/316,399, 08/485,608, 08/442,267, can be used in the formulations.

***B. Preparing the Lipid/Therapeutic Nucleic Acid Particle***

The lipid-nucleic acid formulations can be achieved using any prior art method. The preferred methods for systemic (*i.e.*, intravenous or other parenteral) delivery result in a high-efficiency encapsulation, wherein little of the nucleic acid is exposed to free solution or adsorbed to the outer surface of the lipid particle. Such methods are disclosed in published PCT Patent Application WO 96/40964, U.S. Patent No. 5,705,385, and U.S. Patent Applications Serial Nos. 08/485,458, 08/484,282, 08/660,025, 08/856,374, 60/073,598 and 60/063,473, all of which are assigned to the

assignee of the instant invention and incorporated herein by reference. Generally, high efficiency encapsulation provides low immunogenicity and improved tolerance when injected for systemic delivery. Further, these lipid-nucleic acid particles are relatively easy to characterize and define compared to cationic lipid-plasmid aggregates used in local delivery methods.

Preferred encapsulation methods are set out in the Example section. The lipid-therapeutic nucleic acid particles obtained by these methods have identifiable characteristics which make them suitable for use in the invention. For instance, they are small particles typically having a mean particle size of about 50 to about 200 nm and, preferably, of about 60 to about 130 nm. Most preferably, particles are of a relatively uniform size and have a $\chi^2$ (chi-squared) value of less than about 3, more preferably of less than about 1 and, more preferably, of less than about 0.5.

Moreover, the lipid-therapeutic nucleic acid particles of the present invention are serum-stable and, thus, not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA. Suitable assays for measuring serum stability include a standard serum assay or a DNase assay (which are described in the Example section). Nuclease resistance/serum stability is a measure of the ability of the formulation to protect the therapeutic nucleic acid from nuclease digestion either in an *in vitro* assay or in circulation. The encapsulated particles of the present invention have greater nuclease resistance and serum stability than lipid-plasmid aggregates (also known as cationic complexes), such as DOTMA/DOPE (LIPOFECTIN™) formulations.

In addition, the lipid-therapeutic nucleic acid particles of the present invention have a nucleic acid to lipid ratio that can be formulated at various levels. For use in the methods of this invention, the particles have a drug to lipid ratio of at least about 3 mg of nucleic acid per mmol of lipid, more preferably, at least about 14 mg of nucleic acid per mmol of lipid and, even more preferably, greater than about 25 mg of nucleic acid per mmol of lipid. The preferred particles, when prepared to an administration ready formulation, are about 60 – 80 mg nucleic acid per mmol lipid (*i.e.*, they are "high ratio" formulations). The method used for making high ratio formulations can also be employed using reduced amounts of DNA to obtain lower ratio formulations. As used herein, "drug to lipid ratio" refers to the amount of therapeutic nucleic acid (*i.e.*,

the amount of nucleic acid that is encapsulated and that will not be rapidly degraded upon exposure to the blood) in a defined volume of preparation divided by the amount of lipid in the same volume. This may be determined on a mole per mole basis, on a weight per weight basis, or on a weight per mole basis. For final administration ready formulations, the drug to lipid ratio is calculated after dialysis, chromatography and/or nuclease digestion have been employed to remove as much of the externally associated therapeutic agent as possible. Drug to lipid ratio is a measure of potency of the formulation, although the highest possible drug to lipid ratio is not always the most potent formulation.

An alternative description of the lipid-nucleic acid particles of the present invention is "high efficiency" formulations that emphasizes the active loading process involved and contrasts with low efficiency or passive encapsulation. Passive encapsulation of nucleic acid in lipid particles, which is known in the art, achieves less than 15% encapsulation of therapeutic agent, and results in low ratio particles having less than 3 mg of nucleic acid per mmol of lipid. The preferred lipid/therapeutic nucleic acid particles of the present invention have an encapsulation efficiency of greater than about 30%. As used herein, "encapsulation efficiency" refers to absolute efficiency, *i.e.*, the total amount of DNA added to the starting mixture that ends up in the administration competent formulation. Sometimes the relative efficiency is calculated, wherein the drug to lipid ratio of the starting mixture is divided by the drug to lipid ratio of the final, administration competent formulation. The amount of lipid lost during the formulation process may be calculated. Efficiency is a measure of the wastage and expense of the formulation.

Other beneficial features that flow from the use of the preferred particles of the present invention, such as low nonspecific toxicity, improved biodistribution, therapeutic efficacy and ease of manufacturing, will be apparent to those of skill in the art. It is possible to develop particles as described above by alternative methods of encapsulation. These methods may employ standard techniques for loading of liposomes that are well known for use with conventional drugs. These methods include freeze-thaw extrusion, dehydration/rehydration, reverse phase evaporation, and the like, some of which are disclosed in Monnard, *et al.*, "Entrapment of nucleic acids in liposomes, " *Biochim. Biophys. Acta.*, 1329:39-50 (1997). These methods are not high encapsulation efficiency formulations, nor high ratio formulations, but the instant disclosure suggests the utility of such particles in the use of gene therapy against distal tumor sites.

In addition to the lipids employed in the methods used above, there are a tremendous number of additional lipid and nonlipid components which can be used to enhance delivery or targeting of particles. Additional lipid components include, but are not limited to, lipids with neutral, anionic, cationic or zwitterionic headgroups, and the like. These standard components are set out in the art and in the patent applications referred to above which are incorporated herein by reference. Charged lipids that are particularly preferred with the invention are N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), the subject of recently issued U.S. Patent No. 5,753,613, incorporated herein by reference and assigned to the assignee of the instant invention; and 1,2-Dioleoyl-3-dimethylammonium-propane (DODAP), the subject of U.S. Patent Application Serial No. 08/856,374, the teachings of which are incorporated herein by reference.

Both the nucleic acid and prodrug formulations can include additional components selected from a wide variety of lipids, lipid conjugates and compatible additional components known in the art. For instance, cholesterol and its derivatives can be used in the nucleic acid and prodrug formulations of the present invention. Still other formulations can employ polycationic compounds that can condense DNA into small sizes before lipid encapsulation. Polylysine and polyethyleneimine, among other compounds, have been used by those of skill in the art in this capacity. Condensed particles can also be used in the methods of this invention.

In addition, cloaking agents can be used to reduce elimination by the host immune system. Such cloaking agents include, for example, polyamide oligomer-lipid conjugates, such as ATTA-lipids, disclosed in U.S. Patent Application Serial No. 08/996,783, filed February 2, 1998 (TTC Attorney Docket No. 16303-005800) and PEG-lipid conjugates disclosed in U.S. Patent Application Serial Nos. 08/486,214, 08/316,407 and 08/485,608, the teachings of which are incorporated herein by reference. These components can also be targeting agents that encourage the lipid formulations to accumulate at the area of the disease or target site. In addition, these components can be compounds that improve features of the formulation, such as leakiness, longevity in circulation, reduction in toxicity, encapsulation efficiency, *etc.* Examples of these components and others that can usefully be included in the formulations of the invention are known to and used by those skilled in the art.

With respect to both the nucleic acid and prodrug lipid formulations, it is sometimes preferable to employ a programmable fusogenic lipid formulation. This refers to a formulation that has little tendency to fuse with cell membranes and deliver its payload until a given signal event occurs. This allows the lipid formulation to distribute more evenly after injection into an organism or disease site before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or simply time. In this last event, a fusion delaying or "cloaking" component, such as an ATTA-lipid conjugate or a PEG-lipid conjugate, can simply exchange out of the liposome membrane over time. By the time the formulation is suitably distributed in the body, it is calculated to have lost sufficient cloaking agent so as to be fusogenic. With other signal events, it may be desirable to choose a signal event that is associated with the disease site or target cell, such as increased temperature at a site of inflammation.

One of the great advantages of the invention is its versatility in targeting a broad range of disease sites. In particular, lipid encapsulated formulations are usefully employed in targeting and killing tumor cells and other neoplasia, or other cell types at disease sites that can usefully be modified or sensitized to perform some other function. Other cell types include, but are not limited to, cells at sites of inflammation, sites where genes are aberrantly expressed in sites of infection and the like.

In a preferred embodiment, the nucleic acid, *e.g.*, vector, is delivered in a lipid encapsulated formulation by intravenous administration. This mode of administration takes advantage of the known tendency of lipid encapsulated formulations to accumulate at tumors and neoplasia even without specific targeting aspects. This ability is thought to be the result of "leaky" vasculature at sites of neoplasia which is easily invaded by small sized lipid particles (*see*, Jain, *Sci. Am.*, 271:58-65 (1994)).

Where specific cell type targeting is preferred, the lipid formulation can contain, on the outer surface, antigens or markers that are recognized by moieties or that recognize receptors on the target cell. Examples of such targeting can be found in, for example, Forum, *et al.*, "Liposome Targeting in Animal Models," L. Huang (Ed.), *Journal of Liposome Research*, 7(4):315-534 (1997), the teachings of which are incorporated herein by reference.

## *C. Administration-Ready Pharmaceutical Preparations*

Generally, when administered intravenously, the nucleic acid and/or the prodrug formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. Carriers may also be employed when delivering the vector or prodrug formulations by other parenteral methods known in the art, such as subcutaneous, intratumoral or intramuscular injection, inhalation, and the like.

When preparing pharmaceutical preparations of the lipid/therapeutic nucleic acid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with nucleic acid associated with the external surface.

## *D. Disease Indications Suitable for Treatment by the Invention*

The instant invention is particularly useful for treatment of neoplasia in mammals. Treatment means obtaining a therapeutic effect at the site of neoplasia. Treatment of a diverse range of tumors can be obtained using the compositions of this invention. In addition, the compositions and methods of this invention can be tested

against standard NIH-recommended models. *See*, for example, Driscoll, "The preclinical new drug research program of the National Cancer Institute," *Cancer Treatment Reports*, 68:63-76 (1984). Further, *in vivo* and/or in vitro models that are routinely employed in National Cancer Institute sponsored drug screening evaluations for identifying utility against human neoplasia can be employed to confirm the utility of the instant invention (*See*, Boyd, M.R., "The NCI In Vitro Anticancer Drug Discovery Screen. In Anticancer Drug Development Guide" (B. Teicher (ed.), 1995, Humana Press, Totawa, NJ).

Characteristics of neoplasia against which this invention is useful include, but are not limited to, tumors or neoplasia that are 1) reasonably transfectable by lipid/therapeutic nucleic acid particles; 2) are responsive to the gene product of the nucleic acid; and 3) are not readily accessible to surgical approaches. Such tumors include metastatic or nonsolid tumors, particularly micro-metastases, metastasis found outside of the lung, liver or spleen ("first pass organs"), and the like. Thus, the invention is applicable to a variety of tumor types. A key characteristic of a suitable disease indication will be its accessibility to the lipid/therapeutic nucleic acid particles of the invention.

### *E. Combination Therapy*

Combination chemotherapy, a well-known technique employing conventional drugs for treating cancer, can also be employed using the lipid-nucleic acid particles of the invention. Combination chemotherapy treatment has advantages in that: 1) it avoids single agent resistance; 2) in a heterogenous tumor population, it can kill cells by different mechanisms; and 3) by selecting drugs with nonoverlapping toxicities, each agent can be used at full dose. Combination regimens, which are curative when single agent treatment is not, include, but are not limited to, the following: acute lymphocytic leukemia – vincristine, prednisone, doxorubicin and L-asparaginase; Hodgkin's disease – mechoroethamine, vincristine, procarbazine and prednisone (MOPP); histiocytic lymphoma – cyclophosphamide, vincristine, procarbazine and prednisone (C-MOPP); and testicular carcinoma – bleomycin, vinblastine, cisplatin.

Some considerations that are well known in selecting drug combinations include the following:

Kinetic considerations: A heterogenous tumor will be treated first with noncell cycle specific agents (*e.g.*, cyclophosphamide and doxorubicin) to debulk the

tumor and recruit slowly dividing cells into active DNA synthesis; followed by a cell cycle specific drug, such as methotrexate and 5-FU. The drug cycle is repeated at regular intervals.

Drug resistance considerations: Two or more noncross-resistant drugs are used simultaneously to avoid selection of resistant tumor cells. Double resistant mutants may arise by sequential chemotherapy.

Drug interactions: Some combinations (*i.e.*, methotrexate and 5-FU) are synergistic when given in the proper sequence, antagonistic when the order is reversed. Some combinations (*i.e.*, L-Asparaginase and methotrexate) are antagonistic initially, but after an extended period (about 10 days), tumor cells are found to be more sensitive to methotrexate.

All these considerations may play a role in the proper selection of therapeutic genes and anti-neoplastic agents employed. Some possible combination therapies employing gene therapy and chemotherapeutic agents are set out in U.S. Patent Application Serial No. 60/082,665 (TTC Attorney Docket No. 16303-007100), assigned to the assignee of the instant invention and incorporated herein by reference. It is particularly useful to deliver therapeutic nucleic acids to neoplasia which have been identified as multi-drug resistant, in order to resensitize the tumor to the chemotherapeutic agent.

### *F. Dosages of Drugs*

The precise dosage to be administered to a patient, whether as part of the GDEPT system or as part of combination chemotherapy, will ultimately be dependant upon the discretion and professional judgement of the attendant physician and will be in part dependent on such factors as the age, weight and the particular neoplasia of the patient. The amounts and precise regime will of course depend on other factors including the severity of the condition to be treated.

In other systems, the exact dosage regime will need to be determined by individual clinicians which will be controlled by the exact nature of the nucleic acid to be delivered and the condition to be treated, but some general guidance can be given. In general, dosage can easily range from about 0.1 μg to 1 g or more of nucleic acid. More preferably, the dose of nucleic acid will range from about 0.1 μg to about 5 mg per

kilogram for a typical 70 kilogram patient, and doses of vectors, which include a viral particle, are calculated to yield an equivalent amount of therapeutic nucleic acid.

In GDEPT systems, a suitable dose of the nucleic-acid lipid particle will be the amount of nucleic acid which will produce about 500 to about 200,000 enzyme units/m$^2$ (*e.g.*, 20,000 enzyme units/m$^2$). The dose of the prodrug will advantageously be in the range of about 0.1 to 250 mg per kilogram of body weight of recipient per day, preferably about 0.1 to 100 mg per kilogram bodyweight.

In combination chemotherapy, a suitable dose of the nucleic acid will typically range from about 0.1 μg to about 5 mg per kilogram for a typical 70 kilogram patient. In addition, a suitable dose of the other drug, *e.g.*, the other anti-cancer agent, will advantageously be in the range of about 0.1 to 250 mg per kilogram of body weight of recipient per day and, more preferably, in the range of about 0.1 to 100 mg per kilogram body weight of recipient per day.

Typically, the particle will be administered to the patient and then the uptake and transfection into cells will be monitored, for example by recovery and analysis of a biopsy sample of the targeted neoplastic tissue. This can be determined by clinical trials which involve administering a range of trial dosages to a patient and measuring the degree of transfection in a target cell or tumor. In the methods of the current invention, the prodrug will usually be administered following administration of the nucleic acid encoding a gene product.

The invention will be described in greater detail by way of specific examples carried out in accordance with Canadian Council on Animal Care, 2nd Ed., "Guide to the care and use of experimental animals," Eds. Olfert, E., Cross, B. and McWilliam, A. (1993). The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

# EXAMPLES

## *EXAMPLE 1*

This example illustrates the synthesis of 5 lipid-plasmid particle formulations for systemic delivery.

Materials: Plasmids are preferably supercoiled, 4000 to 15000 bp in length, encoding genes and enhancer elements, *etc.* as desired. Cationic lipid, N,N-dioleyl-N,N-dimethyl ammonium chloride ("DODAC") and monomethoxy polyethylene2000 glycol succinate-(C8:0-ceramide) ("PEG-Cer-C8") were synthesized at Inex Pharmaceuticals Corp. Dioleyl-phosphatidylethanolamine (DOPE) was supplied by Northern Lipids, Vancouver. Standard dialysis membranes: Spectro/Por 5 regenerated Cellulose (12-14,000 MWCO) was purchased from VWR (Manufactured by Spectrum Medical Industries Inc.). Sodium Citrate was purchased from BDH. Sodium Chloride, Triton X-100 and Octyl-beta-D-glucopyranoside ("OGP") were obtained from VWR Scientific, Fisher Scientific or Sigma Chemical Company.

### ***Formulation 1.1 (Or, Alternatively, INEX 303 or INEX 303i)***

Plasmid (50-400 μg) is incubated with DODAC in 500 μL of the prep solution containing 0.2 M OGP in 150 mM NaCl; 5 mM HEPES pH 7.4, for 30 min at room temperature. This mixture is added to a mixture of DOPE and PEG-Cer-C14 or PEG-Cer-C20 or PEG-Cer-C8 in 500 μL of the same prep solution. The total lipid concentration was either 5 or 10 mg/mL, with the molar ratio of DOPE:DODAC:PEG-Cer being 84:6:10. The mixture was dialyzed against 150 mM NaCl; 5 mM HEPES (pH 7.4) for 36-48 h with two buffer changes.

Nonencapsulated DNA was removed by anion exchange chromatography on DEAE-Sepharose column (1 X 4 cm). Empty liposomes were removed by pooling lipid/DNA samples that co-eluted on the DEAE column on top of a sucrose density gradient in 12.5 mL ultracentrifuge tubes. The gradient was formed with 3 mL each of 10% sucrose, 2.5% sucrose and 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 36,000 rpm (160,000 X g) for 2 h at 20°C in a Beckman Optima XL-100K ultracentrifuge using an SW-28 rotor. Separated bands were removed from top to bottom. Fractions were assayed for 3H-plasmid and 14C-CHE by dual-label scintillation counting using a Beckman LS6500 scintillation counter. The lipid

encapsulated plasmid DNA banded tightly at the interface between 2.5% and 10% sucrose, while the unassociated lipid was present as a smear from the top of the gradient to the interface between 1% and 2.5% sucrose. The formulation can be concentrated in 12-14,000 MWCO dialysis tubing against 500,000 MW PEG (Aquacide II). When the desired volume is reached, the formulation was transferred into a new dialysis bag and dialyzed overnight against HBS to adjust the NaCl concentration to 150 mM.

***Formulation 1.2 (Or, Alternatively, INEX 351)***

In formulation 1.2 the following concentrations were used. Lipid concentration: 5.0 mg/mL (or 5.3 mM); plasmid concentration, 200 ìg; initial volume, 1.0 mL; lipid stock solutions (in 95:5 benzene:methanol, 2:1 chloroform:methanol or ethanol). Calculated by molarity (dissolved in 95:5 benzene:methanol or 2:1 chloroform:methanol). DOPE (744 g/mol), 40 mM; DODAC (582 g/mol), 40 mM; and PEG-C8 (2515 g/mol), 20 mM.

**Formulation for 351**: 42.5:42.5:15 (mole %) DOPE:DODAC:PEG-C8

| | DOPE | DODAC | PEG-C8 |
|---|---|---|---|
| mg | 1.68 | 1.315 | 2.005 |
| mole % | 42.5 | 42.5 | 15 |
| μmol | 2.25 | 2.25 | 0.8 |
| μl | 56.2 | 56.2 | 40 |

**Formulation Procedure** (1 ml scale):

Aliquot lipid stock solutions into a clean, dry test tube and dry to a lipid film using a stream of $N_2$ gas and then dry under vacuum for at least 2 hrs. Add 50 μL 2M OGP and add 500 μL of 2X strength dialysis buffer, add 200 μg of plasmid and mix by vortexing to dissolve the lipid film. Make up to 1.0 mL with sterile deionized $H_2O$, mix and allow to incubate approximately 30 min at room temperature. Place the solution into a dialysis bag and dialyze for 40-48 hrs against 2 L of dialysis buffer with 1-2 changes of buffer after approximately 24 hrs, and determine the volume of the sample by weighing in a tarred tube (assume density of 1.0). These steps may be followed by DEAE cleaning and/or sucrose density gradient centrifugation, as described above.

After DEAE cleaning and sucrose density centrifugation, as described above, the final INEX 351 formulation has a concentration of about 200 μg/ml plasmid and 5 mg/ml total lipid.

NOTES for INEX 351:

Note 1: Appropriate dialysis buffer concentrations:

p53 : 150 mM $NaPO_4$ + 150 mM NaCl (try 140 - 160 mM NaCl), pH 7.4

pLuc: + 175 mM NaCl (about 150 - 170 mM NaCl), pH 7.4

Note 2: 150 mM $NaPO_4$ buffer, pH 7.4:

35.77 g dibasic sodium phosphate ($Na_2HPO_4$)

6.62 g monobasic sodium phosphate ($NaH_2PO_4$)

add appropriate quantity of NaCl dissolve in 2 L (final volume) of deionized water with stirring. The final pH may vary between a pH of about 7.3 and about 7.4; this has not normally been adjusted and has not affected the performance of the formulation.

Note 3: Use 0.2 µm filtered buffer with the lipid/plasmid/detergent solution

Note 4: As an alternative to adding 2X dialysis buffer, the plasmid may be pre-dialyzed against dialysis buffer and the formulation may be diluted to its final volume normal strength dialysis buffer. While this means that there will be a slight difference in the buffer concentration, this does not affect the encapsulation efficiency or resulting particle size.

Note 5: If the volume of the formulation is increased (*i.e.*, above 5 mL), add another dialysis change.

Note 6: DEAE-Sepharose columns are often pre-treated by eluting 50 µL of a 10 mg/ml extruded or sonicated 1:1 phosphatidylcholine:cholesterol vesicle formulation (diluted in 2 mL) to block any nonspecific lipid binding to the column.

To reduce the cationic surface charge of INEX 351 formulations, it may be desirable to reduce the amount of cationic lipid (*i.e.*, DODAC) employed. If the amount of DODAC is changed, the amount of DOPE is changed to maintain the same total amount of lipid. Formulations below 30% DODAC are preferably made in 10 mg total lipid. Dialysis buffer may be changed as in Table 1, below:

**Table 1. Characterization of representative large scale formulations.**

| Conc. | Starting volume | Buffer | Encapsulation efficiency | Nicomp particle size (nm)[a] |
|---|---|---|---|---|
| 42.5 % | 30 ml | 150 mM $NaPO_4$, 130 mM NaCl | 49 % | 131 |
| 30% | 12 ml | 150 mM $NaPO_4$ | 56.8 % | 109 |
| 24% | 30 ml | 130 mM $NaPO_4$ | 50.7 % | 250 |
| 20% | 15 ml | 105 mM $NaPO_4$ | 63 % | 178 |

[a]Nicomp analysis of mean particle size, gaussian dist., volume weighting, before DEAE cleaning and isolation.

***Formulation 1.3 (Or, Alternatively, INEX 321)***

Lipid-plasmid particles with 10-30% DODAC are also useful in the present invention. These can be formulated, as described above, or as follows.

Lipid stock solutions: Individual stock solutions of each lipid were dissolved in chloroform/methanol (2:1 v/v) to a final concentration of 2 or 20 mg/mL.

OGP solution: 1.0 M OGP solution was prepared in MilliQ grade water.

Citrate buffer: Sodium citrate buffer was used for dialysis to remove detergent from the formulation. The citrate concentrations were varied according to the amount of DODAC. Buffer also contains 150 mM NaCl and 5 mM HEPES at pH 7.4, unless indicated otherwise. In general, a 10X solution was prepared and diluted 1:10 in MilliQ Plus water for dialysis using a graduated cylinder.

Preparation of lipid/DNA/OGP mixture: A typical formulation contained 10 mg of lipid of DODAC/DOPE/PEG-Cer-C8 and 200 μg DNA. Appropriate amounts of stock solutions containing DODAC, DOPE and PEG-Cer-C8 (normally 15 mol% in this study) were mixed in a glass test tube. If the amount of DODAC is changed, the amount of DOPE is changed to maintain a total of 10 mg lipid. The solvent was first removed under a stream of $N_2$ gas followed by incubation under vacuum for 3-5 h. To the lipid, 0.2 mL of 1 M OGP was added. The suspension was vortexed until the lipid was totally dissolved and the solution became clear. Then a 0.2 mL DNA (1 mg/mL) solution containing 200 μg DNA and 0.6 mL HBS (HEPES buffered saline) or citrate buffer (concentrations designated in Figure 1) were added to a final total volume of 1 mL. If the solution did not become clear, a small amount of OGP (50 μL) was added. The solution was incubated at room temperature for 1 h to allow the components to equilibrate.

Dialysis: Dialysis tubes were soaked in 60% ethanol (or in distilled water if sterilization was not required) for 30 min. The mixture of DNA/lipid/OGP solution was then transferred to the dialysis tube. The sample was dialyzed for 2 days in 2-4 L citrate buffer (concentration as described in Figure 1) with two changes of buffer daily.

After preparation, empty liposomes can be removed by DEAE cleaning and sucrose density centrifugation, as described above. Having been taught the various lipid-plasmid particle formulations suitable for systemic delivery in this example, it would be obvious to one skilled in the art to modify them, for example, for improved plasmid delivery and/or intracellular expression using one or more possible variations. Variations of the following type are suggested: percentage of PEG-lipid; size of PEG; length of hydrophobic (anchor) chain; pH sensitive PEG-lipids; replacement of PEG by

ATTA (disclosed in U.S. Patent Application Serial Number 08/996,783, filed December 22, 1997 (bearing TTC Attorney Docket No. 16303-005800 and which is assigned to the assignee of the instant invention); addition of membrane modifying lipids, such as cholesterol or DOPE; use of alternative cationic lipids, such as DMRIE, DOTAP, DOTMA, DODMA, AL-1, *etc.*; use of fusogenic components, such as pH sensitive lipids, peptides (EALA) or polymers (PEAA); use of targeting agents; use of DNA condensing peptides (*i.e.*, polylysine or spermine) or polymers (*i.e.*, PEI); use of negatively charged lipids, such as phosphatidylserine; or use of alternative PEG-lipid linkers, such as SPDP or PDPH (disclosed in U.S. Patent Application Serial Number 08/536,584, which is assigned to assignee of the instant invention).

***Formulation 1.4***

Formulation 1.4 contains DOPE:DODAC:PEG-Cer-C20 (83:7:10) – mol %. The synthesis protocol is as follows: Aliquot the lipid stock solutions (in ethanol) into an autoclaved, clean, dry round bottom flask. The solution is dried to a lipid film using a rotavap in a 65°C water bath and vacuumed overnight. Add HBS with octylglucopyranoside (OGP) to a final OGP concentration of 200 mM. Swirl the mixture to dissolve the lipid film and, if necessary, heat to 37°C to ensure the lipid is fully dissolved. Plasmid DNA is then added (400 µg / 10 mg lipid) to the dissolved lipid films. After incubation at room temperature for 30 min, place the resulting solution in a dialysis bag that has been pre-soaked in filter sterilized distilled H2O and autoclaved. Dialyze overnight against 20 L of dialysis buffer (5 mM HEPES, 150 mM NaCl, pH 7.4, filter sterilized through a 0.2 micron sterile filter) with two buffer changes.

Nonencapsulated DNA was removed by anion exchange chromatography on a DEAE-Sepharose CL-6B column. Collect the particle suspension as it appears in the eluate, and concentrate using the Amicon difiltration system (YM 30 membrane). Next, empty liposomes were removed using a sucrose density gradient. The gradient was formed by layering 10% sucrose, 5.0% sucrose, and 2.5% sucrose in HBS, pH 7.4. The sample is loaded by floating it on top of the 2.5% sucrose layer and centrifuged at 28,000 rpm for 18 h at 20°C using a Beckman Optima XL-100K ultracentrifuge and an SW-28 rotor. After centrifugation, remove the lower band with a syringe and needle and pool the samples. The sucrose is removed and the sample is concentrated simultaneously using the Amicon system. Filter sterilize the final volume through a 0.2 micron filter. DNA

concentration is analyzed by Picogreen assays, lipid concentration by HPLC and particle size by Nicomp analysis.

***Formulation 1.5***

This method, set out in PCT patent publication WO 96/40964, which is incorporated herein by reference, is an alternative high-efficiency formulation of the lipid/nucleic acid particle. It is, in essence, a preparation of lipid therapeutic nucleic acid particles in organic solvent. The following stock solutions of lipid are prepared in 100% ethanol: DSPC – 20 mg / mL (20 mol%) = 128.4 μL; Chol – 20 mg / mL (25 mol%) = 113.1 μL; DODAP – 40 mg/mL (45 mol%) = 44.5 μL; PEG-Cer-C20 (or C14) – 50 mg/mL (10 mol%) = 67.6 μL.

The lipids are mixed together and the volume is increased to a total volume of 0.400 mL with 100% ethanol. An appropriate volume of 300 mM citrate buffer (pH 3.3) is added to the DNA to a final volume of 600 μL and pH 3.8. Warm the two solutions to 65°C for 2 min. While vortexing the DNA tube, use a Pasteur pipette to add lipid (in ethanol) in a dropwise manner to the DNA solution. The resulting solution will get cloudy and can bubble, but no aggregates should be present. Place the solution in presoaked dialysis tubing (12-14,000 MWCO) and dialyze for 1 h against 300 mM citrate buffer (pH 3.7-4.0). Transfer the dialysis tubing to HBS (pH 7.5) and dialyze for 12 h. Nonencapsulated DNA was removed by anion exchange chromatography using a DEAE-sepharose column equilibrated in HBS. If necessary, the final preparation can be concentrated using the Amicon system (YM 30 membrane). DNA concentration is analyzed by Picogreen assays and the lipid concentration by HPLC.

All of the above lipid-therapeutic nucleic acid formulations have beneficial characteristics that make them suitable for use in the methods of the present invention. Such characteristics include, but are not limited to, the following: First, they are small particles with mean sizes of about 50 about 200 nm and, more preferably, of about 60 to about 130 nm. Most preferably, particles are of a relatively uniform size and have a î2 value of less than 3, more preferably, of less than 1 and, even more preferably, of less than 0.5. Second, they are serum-stable and, thus, are not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA. Third, they have a nucleic acid to lipid ratio that can be formulated at various levels. For use in

the methods of this invention, particles are preferably at least about 3 mg nucleic acid per mmol lipid, more preferably at least about 14 mg per mmol lipid and, most preferably, greater than about 25 mg per mmol. The lipid-nucleic acid particles of the present invention have other advantageous features, such as low nonspecific toxicity, improved biodistribution, therapeutic efficacy and ease of manufacturing.

*Assays for Serum Stability*

Lipid/therapeutic nucleic acid particles formulated according to the above noted techniques can be assayed for serum stability by a variety of methods.

For instance, in a typical DNase 1 digestion, 1 µg of DNA encapsulated in the particle of interest is incubated in a total volume of 100 µL of 5 mM HEPES, 150 mM NaCl, 10.0 mM MgCl2 pH 7.4. DNase treated samples are treated with either 100 or 10 U of DNase I (Gibco – BRL). 1.0 % Triton X-100 can be added in control experiments to ensure that lipid formulations are not directly inactivating the enzyme. Samples are incubated at 37°C for 30 min after which time the DNA is isolated by addition of 500 µL of DNAZOL followed by 1.0 mL of ethanol. The samples are centrifuged for 30 min at 15,000 rpm in a tabletop microfuge. The supernatant is decanted and the resulting DNA pellet is washed twice with 80% ethanol and dried. This DNA is resuspended in 30 µL of TE buffer. 20 µL of this sample is loaded on a 1.0% agarose gel and subjected to electrophoresis in TAE buffer.

In a typical serum assay, 50 µg of DNA in free, encapsulated, or encapsulated + 0.5% Triton X100 was aliquoted into 1.5 mL Eppendorf tubes. To the tubes were added 45 µl normal murine or human serum, $dH_2O$ (to make final volume 50 µL). The tubes were sealed with parafilm and incubated at 37°C. A sample of the free, encapsulated, or encapsulated + 0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an Eppendorf tube and stored at -20°C. Aliquots were taken at various time points, added to GDP buffer containing proteinase K (133 µg/mL) and immediately frozen in liquid nitrogen to stop the reaction. Once all of the time points were collected, the samples were incubated at 55°C in a waterbath to activate proteinase K enabling it to denature any remaining exonuclease. Proteinase K digested samples were applied to polyacrylamide gels to assess levels of exonuclease degradation.

Particles disclosed above demonstrate serum stability by showing less than 5% and preferably undetectable amounts of DNA degradation (partial or total) as a result of such treatment, even in the presence of 100 U DNase 1. This compares favorably to free DNA, which is completely degraded, and plasmid/lipid complexes (such as DOTMA or DODAC:DOPE complexes), wherein DNA is substantially (*i.e.*, greater than 20%, often 80%) degraded after such treatment.

***EXAMPLE 2***

This example illustrates the measurement of the therapeutic effect of lipid formulated ganciclovir on subcutaneous tumors transfected with lipid encapsulated HSV-TK.

| Group | Tumor | Plasmid | Prodrug | Route | Assay | Mice per Group |
|---|---|---|---|---|---|---|
| A | B16 | L018 | PBS | IV | Tumor Volume | 6 C57 |
| B | B16 | L018 | GCV | IV | Tumor Volume | 6 C57 |
| C | B16 | pTK010 | PBS | IV | Tumor Volume | 6 C57 |
| D | B16 | pTK010 | GCV | IV | Tumor Volume | 6 C57 |

On day zero, 24 female C57 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) are seeded sub-cutaneously with 100,000 B16 mice melanoma cells (NCI catalog B16BL-6) in a total volume of 50 μL (groups A, B, C, D). Tumor volume is determined daily by measuring the length, width and height of the tumor with skin calipers as soon as possible and every day thereafter. Groups A to D are treated with 100 μg plasmid of the appropriate lipid-formulated plasmid, formulated according to Example 1, once daily beginning at 9:00 a.m. on day five and on every day following. The plasmid formulation is injected IV in the tail vein in a total volume of 200 μL PBS. Groups B and D are treated with lipid formulated ganciclovir, prepared according to Example 4, once daily beginning at 3:00 p.m. on day five and on every day following. 0.5 mg ganciclovir (~25 mg/kg) are injected IV in the tailvein in a total volume of 200 μL PBS. On day 21, mice are sacrificed. Tumors are collected and weighed.

The results obtained demonstrate that the mice of group D either did not develop tumors, or else developed tumors significantly more slowly than mice of control groups A, B and C.

***EXAMPLE 3***

This example illustrates the measurement of therapeutic effect of systemic delivery of treatment with the therapeutic nucleic acid HSV-TK followed by treatment with lipid-formulated ganciclovir on SCID mice having human (SKOV-3) intraperitoneal (IP) tumors.

| Group | Tumor | Plasmid | Prodrug | Route | Assay | Mice per Group |
|---|---|---|---|---|---|---|
| A | SKOV-3 | L018 | PBS | IV | Tumor Volume | 6 C57 |
| B | SKOV-3 | L018 | GCV | IV | Tumor Volume | 6 C57 |
| C | SKOV-3 | pTK010 | PBS | IV | Tumor Volume | 6 C57 |
| D | SKOV-3 | pTK010 | GCV | IV | Tumor Volume | 6 C57 |

* It is noted that the "Route" refers to the delivery of the prodrug.

On day zero, 24 female C57 mice are seeded intraperitoneally with 5,000,000 SK-OV-3 human ovary adenocarcinoma cells (ATCC HTB-77)) in a total volume of 500 μL (groups A, B, C, D). Groups A to D are treated with 100 μg plasmid of the appropriate lipid-formulated plasmid, formulated according to Example 1, once daily beginning at 9:00 a.m. on day five and on every day following. The plasmid formulation is injected IV in the tail vein in a total volume of 200 μL PBS. Treatment continued for two weeks.

Groups B and D are treated with lipid formulated ganciclovir, prepared according to Example 4, once daily beginning at 3:00 p.m. on day five and on every day following. 0.5 mg ganciclovir (~25 mg/kg) are injected IV in the tailvein in a total volume of 200μL PBS. Mice are monitored for survival. If tumors develop, mice are sacrificed and the tumors are collected and weighed. The results obtained demonstrate that the mice of group D either did not develop tumors, or else developed tumors significantly more slowly than mice of control groups A, B and C.

***Example 4***

This example illustrates the protocol for the preparation of lipid formulated ganciclovir in a sphingomyelin/cholesterol lipid formulation.

For a 1 mL preparation use 100 mg (180 μmole) of lipid, of which 55 mole% is sphingomyelin (99 μmoles) and 45 mole% (81 μmoles) is cholesterol (Northern Lipids, Vancouver, BC). Dissolve each lipid in 1 mL of chloroform. Aliquot the required amounts of each lipid into one tube to obtain a 55/45 SM/Chol mixture. Next, add 4500 dpm/μmole of lipid of $^{14}C$-CHE ($^{14}C$-cholesteryl hexadecyl ether) and dry the lipid to near dryness under nitrogen. Apply to the lyophilizer overnight and make up a 70/30% solution of HBS/ethanol (HBS is 20 mM Hepes, 145 mM NaCl, pH 7.45). Next, dissolve 100 mg ganciclovir (109 mg ganciclovir-Na, Hoffman LaRoche Ltd.) in 1 mL of 70/30% HBS/ethanol and vortex well. Add 42000 dpm/μmole 3H-GCV (7.5 μL of a 1 Ci/mL stock) and add ganciclovir solution to the lipid film and vortex well. Vortex until the solution appears homogeneous. Freeze-thaw for 5 cycles (liquid nitrogen and 65°C). Warm the cryovial up slightly before putting in the water bath. Next, take 2-10 μL pre-extrusion samples and assay for radioactivity using the dual label program. Take note of the final volume and use this to determine specific activity for both the lipid and GCV. Extrude the sample 2 x through 3 x 100 nm filters at 65°C at 350 psi. At this point, the sample becomes quite viscous. Add 1 mL HBS to the samples and mix by pipetting up and down. Continue extrusion for a total of 10 passes. Take 2-10 μL post-extrusion samples and assay for radioactivity. Hydrate some dialysis tubing (MW cutoff 12,000-14,000) in $dH_20$ for 15 min. Next, put the extruded sample in the tubing and dialyze for 1 h against 2 L HBS. Change to fresh buffer and dialyze overnight. Take 2-10 μL samples and assay for radioactivity and determine the percentage loading by comparing the pre-extrusion and post-dialysis ratios of $^3H/^{14}C$. For example: $^3H/^{14}C$ pre-extrusion = 12.0; $^3H/^{14}C$ post-dialysis = 1.2; 1.2/12.0 x 100% = 10% encapsulation.

***EXAMPLE 5***

This example sets forth the protocol for stable transfection of B16 tumor cells with HSV-TK, for use in Examples 6 and 7, as described in Short Protocols in Molecular Biology, Third Edition, page 9-13 to 9-15, with the following modifications.

According to the method, the following materials were used. Plasmids: pCMVTKIRESneo is based on plasmid pBR322 and includes a CMV promoter, HSV-TK gene, internal ribosome entry site and neomycin resistance gene. L018 is also based on pBR322, but carrying a CMV promoter and a luciferase gene. First, plate B16 murine

melanoma cells in a tissue culture flask (T-75) at 5 x $10^5$ cells/flask in 10 mL MEM media with addition of 10% FBS and Glutamine and grow overnight in $CO_2$ incubator at 37°C to 70% confluency. Next, aspirate media and feed cells with 3.8 mL fresh media per flask 2 h prior to transfection and prepare plasmid/lipid Lipofectin (GIBCO BRL) aggregate in a polystyrene tube according to manufacturer's instructions. Alternatively, dilute plasmid to 20 μg/mL in sterile distilled water. Use Luciferase L018 plasmid as a control for negative selection in Geneticin (G418), use Thymidine Kinase (neomycin) 20A for TKneo stable cells; dilute lipid to 1 mM in sterile distilled water; dilute lipid to charge ratio 1 in sterile distilled water (1.2 mL lipid/8 mL water); add volume of DNA (20 mg/mL) to equivalent volume of lipid (CR1) dropwise while vortexing; and incubate DNA/lipid complex for 30 min at room temperature. Next, slowly add 1.2 mL DNA/lipid complex/T75 flask, mix gently and incubate 24 h in $CO_2$ incubator at 37°C (complex is diluted 1:4 in media). Aspirate media, wash with PBS buffer and split each T75 flask into 2-100 x 20 mm tissue culture dishes. Next, 24 h after plating into dishes, add the selective agent, Geneticin(G418), at the appropriate concentration to kill nontransfected cells, yet allow cells with transfected TKneo to stay alive. The Luciferase control cells should die. Every 2-3 days change the media to remove dead cell debris. Within 10 days clones are visible on bottom of 100 mm dish which are neomycin resistant and TK positive. Scrape clones into 1 mL media in 24-well plate and expand up into T-75 flask. Cells that stably express TK can then be used for local, regional or systemic injection into mice. To screen identified clones for TK expression, plate 2000 cells/well in 96 well plate in 150 μL volume and grow 48 h in $CO_2$ incubator at 37°C; add the specific prodrug for TK, ganciclovir, in a dilution series across the plate made up at 2.5 x concentrated and add 100 μL/well (add to 150 μL volume); incubate 3 days in $CO_2$ incubator at 37°C; aspirate media from wells and add Alamar Blue as per manufacturers instructions (Biosource International) (1:10 dilution in media); and 100 μL/well and incubate for 1, 2, 4 h and read plate at time intervals on fluorescent plate reader (550, 595 nm; 750V; 70 offset; 100 ms integration time).

*EXAMPLE 6*

This example illustrates the effects of systemically delivered lipid-formulated ganciclovir on tumor growth in mice having B16 intradermal tumors stably transfected with HSV-TK.

| Group | Tumor | Prodrug | Route | Assay | Timepoint (assay) | Mice per Group |
|---|---|---|---|---|---|---|
| A | B16 | PBS | IV | Tumor Volume | DAILY | 8 C57 |
| B | B16 TK | PBS | IV | Tumor Volume | DAILY | 8 C57 |
| C | B16 | LIPO-GCV | IV | Tumor Volume | DAILY | 8 C57 |
| D | B16 TK | LIPO-GCV | IV | Tumor Volume | DAILY | 8 C57 |

* It is noted that the "Route" refers to the delivery of the prodrug, *i.e.*, gangciclovir (GCV).

32 female C57 mice (Harlan Sprague Dawley, Inc., Indianapolis) were seeded intradermally in groups A and C with B16 parental control cells, and in groups B and D with B16 tumor cells stably transfected and expressing HSV-TK (prepared as in previous described) at a dose of 150,000 cells in a total volume of 50 μL phosphate buffered saline on day zero. Intradermal tumor volume was determined daily by measuring the length, width and height of the tumor with skin calipers as soon as possible and every day thereafter.

The mice were treated with the ganciclovir prodrug, lipid formulated as in Example 4, once every two days beginning on day four and on every second day following. The ganciclovir dosage of 0.5 mg (~25 mg/kg) was injected IV in a total volume of 200 μL PBS (phosphate buffered saline). Mice received a total of nine treatments. On day 21, mice were sacrificed. Tumors were collected and weighed prior to fixation for sectioning.

Intradermal tumors stably transfected with HSV-TK showed no measurable growth when treated systemically with lipid formulated ganciclovir. Untreated B16 tumors, and treated B16 tumors without TK, were not effected by the drug.

*EXAMPLE 7*

This example was carried out to determine the effect of lipid formulated ganciclovir on TK gene expression in B16 tumor cells stably transfected with HSV-TK and implanted intravenously.

| Group | Tumor | Prodrug | Route | Assay | Timepoint (assay) | Mice per Group |
|---|---|---|---|---|---|---|
| A | B16 TK | PBS | IV | Tumor Volume | DAILY | 8 C57 |
| B | B16 TK | LIPO-GCV | IV | Tumor Volume | DAILY | 8 C57 |

* It is noted that the "Route" refers to the delivery of the prodrug, *i.e.*, gangciclovir (GCV).

16 female C57 mice were seeded with B16 tumor cells stably expressing HSV-TK by tail vein injection at a dose of 150,000 cells in a total volume of 200 μL phosphate buffered saline on day zero. The mice were treated with the ganciclovir prodrug, lipid formulated as in Example 4, once every day beginning on day two and on the two days following. The ganciclovir dosage of 0.5 mg (~25 mg/kg) was injected IV in a total volume of 200 μL PBS (phosphate buffered saline). Mice received a total of three treatments. On day 21, mice were sacrificed and tumors were scored. Livers, lungs, spleen and pancreas were photographed. There was a significant reduction in both size and number of metastatic tumor nodules.

*EXAMPLE 8*

This example illustrates gene expression in distal metastatic tumors using Formulation 1.1 lipid plasmid particles.

On day zero, C57BL/6 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) are seeded with 300,000 LL/2 (Mouse Lewis Lung Carcinoma) cells (ATCC CRL-1642) by intravenous/tail vein injection with total volume 200 μL. On day 10, the mouse is intravenously injected with formulation 1.1 plasmid-lipid particles. The particles carry plasmid L018, which is a standard construct containing the luciferase gene linked to the CMV promoter. At various time points after plasmid injection, mice are sacrificed, and organs and tumors are quickly frozen in liquid nitrogen, then stored at –70°C. Organs/tumors are assayed for the luciferase gene to demonstrate delivery to the

organ/tumor site. Biodistribution results for organs are shown in Figure 2. Accumulation at the tumor site is illustrated in Figure 3. Southern blot data shows presence of intact plasmid at the tumor site increasing to at least 96 h. Cell protein from organs/tumors is also prepared and assayed for luciferase according to standard techniques. A time course of gene product activity at distal (metastatic) tumor sites is demonstrated in Figure 4.

***EXAMPLE 9***

This example illustrates the systemic vector delivery and gene expression in an *in vivo* human tumor.

SCID mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) are seeded with 1 x $10^6$ LS180 human colon adenocarcinoma cells (ATCC CL-187) by subcutaneous injection on day zero. On day 11, mice in groups A, B and C are injected intravenously with indicated doses of L018 plasmid in lipid formulation 1.1, in 200 μL total volume. On day 17, mice in group D and E are injected intravenously with L018 plasmid in lipid Formulation INEX 320, using C8 or C20 PEG-Cer) according to Example 1, in 200 μL total volume. At the times indicated after plasmid injection, mice are sacrificed and organs (liver, spleen and lungs) and tumors are harvested. Expression of the enzyme Luciferase is assayed according to standard techniques on all samples.

The data obtained demonstrates the excellent transfection and expression of the reporter gene Luciferase achieved in an *in vivo* human tumor using the lipid-nucleic acid particles of the present invention (*see*, Figure 5).

| Group | Formulation | Assay | Time Point | Mice per group |
|---|---|---|---|---|
| A | 1.1 (75 μg) | Luciferase | 48 h | 5 |
| B | 1.1 (100 μg) | Luciferase | 48 h | 5 |
| C | 1.1 (125 μg) | Luciferase | 48 h | 5 |
| D | 320 (100 μg) | Luciferase | 24 h | 4 |
| E | 320 (100 μg) | Luciferase | 48 h | 4 |
| F | PBS | Luciferase | 48 h | 1 |

*EXAMPLE 10*

This example demonstrates systemic delivery and expression at an *in vivo* tumor site of a vector containing the HSV-TK gene, using a lipid-nucleic acid particle prepared according to Example 1.

C57 mice are intraperitoneally seeded with 100,000 B16 tumor cells in a total volume of 200 μL PBS on day zero. On day 14, test mice are injected with Formulation 1.2 (100 μg DNA in 500 μL PBS) prepared according to Example 1. The plasmid vector used is pINEX-TK10 as described earlier. 24 h later, mice are sacrificed, and tumors are isolated, fixed within 5 min, and prepared in paraffin sections using standard techniques. The expression of the HSV-TK gene at the distal tumor site is assayed by in situ RNA/RNA hybridization using techniques standard in the art. One such technique is summarized below.

The pattern of HSV-TK gene expression within peritoneal tumors is demonstrated in Figures 6(A) and (B). In all cases of gene expression, positive signal is observed as a cellular content of B16 cells or endothelial cells. Positive stained cells are localized in proliferative zone associated with blood vessels or peripheral area.

*EXAMPLE 11*

This example describes the treatment of tumors using the method of the invention. In particular, this example demonstrates the effect of pINEX-TK10 in Formulation 1.1, in inhibiting the growth of MCA-207 tumors following treatment with ganciclovir. The general method is set out in Figure 8.

The pINEX-TK10 construct consists of a pBR322 derived plasmid containing a CMV promoter linked to a “hyper” HSV-TK gene, bovine growth hormone

polyadenylation sequence and kanamycin resistance gene. “hyper” HSV-TK is a more active form of the HSV-TK gene as disclosed by Black, *et al.*, *PNAS (USA)*, 93:3525-3529 (1996). The plasmid construct employed is set forth in Figure 7.

| Group | Formulation | GCV | Route* | Assay | Timepoint | # of Mice |
|---|---|---|---|---|---|---|
| A | PBS | PBS | IP | Volume/CTL | --- | 6 C57 |
| B | Empty 1.1 | PBS | IP | Volume/CTL | --- | 6 C57 |
| C | 1.1 TK | PBS | IP | Volume/CTL | --- | 6 C57 |
| D | 1.1 TK | GCV | IP | Volume/CTL | --- | 6 C57 |

*It is noted that the "Route" refers to the delivery of the prodrug, *i.e.*, gangciclovir.

24 female C57 mice were seeded with 100,000 MCA-207 fibrosarcoma tumor cells (provided by S. Rosenberg, National Cancer Institute, Frederick/Bethesda, MD) by intra-dermal injection on day zero. The tumor cells had been cultivated and prepared according to standard techniques using RPMI media with 10% Fetal Bovine Serum (*see* for example Current Protocols in Molecular Biology). Beginning on day 5, all animals were treated with the lipid/therapeutic nucleic acid formulation listed in the chart, *supra*. The formulation was delivered intravenously through the tail vein. 80 μg of pINEX-TK10 DNA were injected in a total volume of 200 μL. Treatments were administered on days 5, 7, 9, 11 and 13.

Beginning on day 5, all animals were treated with ganciclovir twice daily. 1 mg (~50 mg/kg) were injected intra-peritoneally in a total volume of 200 μL PBS. Treatments continued twice daily for 12 days (*see*, Figure 9(A)). Mice were monitored for tumor growth.

Figure 9(B) sets out in more quantitative terms the effect of the treatments. Mice treated with HSV-TK in formulation 1.1 have greatly reduced tumors compared to control treated mice. Not shown is data of control mice which demonstrates that treatment with empty liposomes and ganciclovir has no effect on tumor reduction. Figure 9(C) demonstrates the effect of the treatment on test mice in comparison with control mice at day 16 after tumor inoculation.

*EXAMPLE 12*

This example illustrates the protocol for RNA/RNA *in situ* hybridization of *in vivo* tumors transfected by systemically delivered plasmid.

Tumors are prepared for *in situ* investigation by paraffin embedding and staining. Specifically, peritoneal tumors are collected into 4% paraformaldehyde/PBS fixative (Sigma Chemical Co.) and fixed overnight at 4°C. Serial 5-μm sections are prepared after the samples have been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in paraffin wax (Paraplast Plus, Fisher).

When ready to be used, prepared samples are treated with two changes of xylene for 10 min, each followed by rehydration in graded ethanol solutions to 50% ethanol. Samples are prehybridized by standard rinsing, incubation with 0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride, followed by treatment at 56°C for at least 60 min in hybridization buffer containing: 40% deionized formamide, 10% dextran sulfate, 1 x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10 mg/mL RNase-free bovine serum albumin), 4 x SSC, 10 mM DTT, 1 mg/mL yeast t-RNA, and 1 mg/mL denatured and sheared salmon sperm DNA.

Labelling of RNA probe by in vitro transcription of DNAwas as follows. The fragment of 599 bp (532 - 1131) from HSV-TK (pTK10) was cloned into KpnI and BamHI sites of the vector pGEM-7Zf(+) (pTK11). The plasmid is cloned by standard techniques and prepared using Qiagene 500 (Qiagen, Inc.). For the anti-sense probe, this plasmid is linearized by cutting it with KpnI at the original 5' end of the cDNA HSV-TK and purified. The same logic is used for sense (control) probe (*i.e.*, cut at the side of the 3-end of insert by BssHII or BamH or SacI). The plasmid is purified by ethanol precipitation. The following are then mixed in a 1.5 mL sterile RNase free microcentrifuge tube on ice: 4 μL (4 μg) purified, linearized plasmid DNA, 5 μL of 10 x concentrated DIG RNA Labeling Mix (supplied by manufacturer), 10 μL 5 x concentrated Transcription Buffer (400 mM Tris-HCl (pH 8.0, 20°C), 60 mM MgCl2, 100 mM Dithiothreitol (DTT), 20 mM spermidine], 2 μL RNasin, 3 μL RNA polymerase (SP6 for antisense or T7 for sense), and sterile, redistilled water to make a total reaction volume of 50 μL.

The components are mixed and centrifuged briefly, and then incubated for 2 h at 37°C (for T7 RNA polymerase) or at 40°C (for SP6 polymerase). After incubation,

add 3 μL DNase I, RNase free and 1 μL RNasin to the tube and incubate for 15 min at 37°C. Then add 2.5 μL 0.5 M EDTA (pH 8.0) to the tube to stop the polymerization reaction.

The labeled RNA transcript is precipitated by performing the following steps. Add to the reaction tube 6.25 μL 4 M LiCl and 180 μL prechilled (-20°C) 100% ethanol incubate overnight at -70°C. Centrifuged the tube (at 13,000 x g) for 15 min at 4°C. Discard the supernatant. Wash the pellet with 50 μL ice-cold 70% (v/v) ethanol. Centrifuge the tube (at 13,000 x g) for 5 min at 4°C. Discard the supernatant and dry the pellet at room temperature. Dissolve the RNA pellet for 30 min at 37°C or (R.T.) in 20 μL DEPC (diethylpyro-carbonate)-treated, sterile, redistilled water, added 20 μL deionized formamide and 1 μL RNasin. Keep transcript at -20°C or -70°C.

An accurate quantification of DIG-labeled RNA obtained in the labeling reaction is most important for optimal and reproducible results in various membrane or in situ hybridization techniques. Too high of a probe concentration in the hybridization mix usually causes background, while too low of a concentration leads to weak signals. The estimation of yield can be performed in a side by side comparison of the DIG-labeled sample nucleic acid with a DIG-labeled control, that is provided in the labeling kits. Dilution series of both are prepared and spotted on a piece of membrane. Subsequently, the membrane is colorimetrically detected. Direct comparison of the intensities of sample and control allows the estimation of labeling yield.

The hybridization reaction is then performed. Drain pre-hybridization buffer from the pre-hybridized slides and overlay each section with 200 μL of hybridization buffer containing 0.2-1 ng of digoxigenin-labeled RNA probe (0.2 ng/μL). Cover samples with a 24 x 30 mm hydrophobic plastic coverslip. Incubate sections at 56°C overnight in a humid chamber. Washes can include an RNAse step which reduces the background, but decreases the signal as well. It is important to keep the tissue sections moist at all times during washing. Wash the slides in 2xSSC at 55°C for 30 min; wash in 50% formamide, 2xSSC at 65°C for 30 min; wash in 2xSSC 3 times at 37°C for 5 min each; wash in RNase 10 μg/mL, 1xwashing solution at 37°C for 30 min; wash in 50% formamide, 2xSSC at 65°C for 30 min; wash in 2xSSC at 37°C for 15 min; wash in 02xSSC 5 times at 37°C for 5 min each.

After hybridization, cells are incubated DIG-specific antibody. Wash the slides in TBS at RT for 30 min. Incubate sections with blocking solution (TBS and 2% goat serum) at RT for 1 h. Decant blocking solution and incubate sections with goat anti-DIG-alkaline phosphatase (Fab fragment) dilution 1:500 at RT for 1 h. Wash the slides in TBS at RT for 30 min. Wash the slides in substrate buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$) at RT for 30 min. Prepare a color solution containing: 10 mL substrate buffer, 50 µL NBT (nitroblue tetrazolium) and 37 µL BCIP. Slides are immersed in color solution at room temperature for 1-2 h or at 4°C for 2-3 days. Slides are washed with water and observed by light microscopy. Results are shown in Figures 6(A) and (B).

## *EXAMPLE 13*

This example illustrates the treatment of tumors using the methods and compositions of the invention. In particular, this example demonstrates the long-term survival of mice bearing MCA-207 tumors that were treated with a course of intravenous pINEX-TK10 in Formulation 1.1 combined with intra-peritoneal injections of ganciclovir (GCV).

The pINEX-TK10 construct, as described above, was formulated in Formulation 1.1, as described above. C57 mice were seeded with 100,000 MCA-207 fibrosarcoma tumor cells (provided by S. Rosenberg, National Cancer Institute, Frederick/Bethesda, MD) by intra-dermal injection on day zero. The tumor cells had been cultivated and prepared according to standard techniques using RPMI media with 10% Fetal Bovine Serum (*see* for example Current Protocols in Molecular Biology).

There were a total of 20 mice in each of the treatment groups and the groups were treated as follows: Group I, ganciclovir alone (GCV); Group II, empty SPLPs and ganciclovir (Empty SPLP + GCV); Group III, formulated plasmid alone (INXC-gTK); and Group IV, formulated plasmid together with ganciclovir (INXC-gTK + GCV). The lipid/therapeutic nucleic acid formulation was delivered intravenously through the tail vein. 80 µg of pINEX-TK10 DNA were injected in a total volume of 200 µL PBS. Treatments with the lipid/therapeutic nucleic acid formulation were administered on days 5, 7, 9, 11 and 13. Animals were treated with ganciclovir twice daily. 1 mg (~50 mg/kg) were injected intra-peritoneally in a total volume of 200 µL

PBS. Treatments continued twice daily for 12 days. Mice were monitored for long-term survival.

Figure 10 sets out in quantitative terms the effect of the treatments. Mice treated with HSV-TK in Formulation 1.1 together with ganciclovir have greatly increased long-term survival. Only modest effects were observed following treatment with HSV-TK in formulation 1.1 alone compared to untreated controls. Such results demonstrate the successful use of a nonviral gene transfer system that can produce regression in distal tumors following intravenous delivery.

***EXAMPLE 14***

This example illustrates the efficacy of systemic delivery of TK303 in the BALB/c CT26 tumor model, *i.e.*, a colorectal tumor model.

| Group | Formulation | GCV | Route | Assay | # of Mice |
|---|---|---|---|---|---|
| A | HBS | PBS | IP | Volume / CTL | 8 BALB/c |
| B | Empty 303 | PBS | IP | Volume / CTL | 8 BALB/c |
| C | Empty 303 | GCV | IP | Volume / CTL | 8 BALB/c |
| D | 303 TK | PBS | IP | Volume / CTL | 8 BALB/c |
| E | 303 TK | GCV | IP | Volume / CTL | 8 BALB/c |

On day zero, 48 female BALB/c mice were seeded with 100,000 CT26 tumor cells (ID) using the protocol described in the above examples. Beginning on day 5, all animals were treated with formulation delivered IV through the tail vein. 50 μg of DNA was injected IV in a total volume of 200 μL PBS. Treatment was continued every other day for the next eight days.

Groups C and E were treated with lipid formulated ganciclovir once daily on day five and on every day following. 1.5 mg (~75 mg/kg) was injected IV in the tail vein in a total volume of 300 μL PBS. Mice were monitored for survival. If tumors developed, mice were sacrificed and the tumors collected and weighed. The results obtained demonstrate that the mice of Group E either did not develop tumors or else developed tumors significantly more slowly than the other mice (*see*, **Figure 11**).

*EXAMPLE 15*

This example illustrates the efficacy of systemic delivery of TK303 in the BALB/c CT26 tumor model, *i.e.*, a colorectal tumor model.

| Group | Formulation | GCV | Route | Assay | # of Mice |
|---|---|---|---|---|---|
| A | HBS | PBS | IP | Volume | 8 BALB/c |
| B | Empty 303 | PBS | IP | Volume | 8 BALB/c |
| C | Empty 303 | GCV | IP | Volume | 8 BALB/c |
| D | 303 TK FS* | PBS | IP | Volume | 8 BALB/c |
| E | 303 TK FS* | GCV | IP | Volume | 8 BALB/c |
| F | 303 TK | PBS | IP | Volume | 8 BALB/c |
| G | 303 TK | GCV | IP | Volume | 8 BALB/c |

* The vectors used in this experiment were as follows: "TK" has the thymidine kinase coding sequence in its proper reading frame, such that thymidine kinase protein is expressed. "TK FS" has the thymidine kinase coding sequence out of frame relative to the translation initiation codon so that no thymidine kinase protein is produced.

On day zero, 65 female BALB/c mice were seeded with 100,000 CT26 tumor cells (ID) using the protocol described in the above examples. Beginning on day 5, all animals were treated with formulation delivered IV through the tail vein. 50 μg of DNA was injected IV in a total volume of 200 μL PBS. Treatment was continued every other day for the next eight days.

Groups C, E and G were treated with lipid formulated ganciclovir once daily on day five and on every day following. 1.0 mg (~50 mg/kg) was injected IV in the tail vein in a total volume of 200 μL PBS. Mice were monitored for survival. If tumors developed, mice were sacrificed and the tumors collected and weighted. Mice in Group G, which received GCV and the lipid formulated TK construct, and thus expressed the TK protein, exhibited a marked reduction in tumor growth rate (*see*, **Figure 12**).

It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which

such claims are entitled. The disclosures of all articles and references, including patent applications and publications, are incorporated herein by reference for all purposes.